

PRODUCT DERIVED FROM MILK SUBSTANTIALLY FREE OF BETA CASEIN FROM NON-HUMAN MAMMALS AND RELATIVE USE

Field of invention

The present invention is related to a product derived from milk  
5 substantially free of beta casein from non-human mammals. The invention is also related to the use of such a product especially in terms of its application in relation to diet, particularly for early infancy, in the prevention of insulin-dependent diabetes.

Prior art

10 The technique of obtaining products, especially food products, for early infancy is well-known, starting from non-human milk, such as cow's, sheep and goat's milk. The basic component of milk is characterized by casein, which in basic terminology represents a group of proteins obtainable by milk precipitation at acid pH and up to room  
15 temperature, specifically pH 4.6 and 20°C. Caseins represent approximately 80% of total cow's milk proteins and 40% p/v human milk. Casein can be sub-divided into three main groups: alpha, beta and kappa. There is also a fourth group, represented by gamma casein, which is derived from beta casein following the removal of the first  
20 twenty-two amino acids. Therefore, for the present invention, gamma casein will be considered as part of beta casein.

Beta casein represents approximately 70% p/v of all casein present in human milk, whereas in cow's milk, it represents approximately 25% p/v. Of bovine beta casein, several genetic variants are known and have  
25 been characterized, including A1, A2, A3, B, C, D and E. For the industrial production of milk, mainly the genetic variant of milk A1 has been favored to increase the amount of milk produced. This

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## Summary of the invention

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Another object of the invention is a milk-derived product or milk

itself comprising at least one beta casein modified from non-human mammals without the immunogenic characteristic mentioned above.

Another object of the invention is the use of such a product, in relation to diet.

- 5 Another object of the invention is the use of a product from milk or milk itself, substantially free of non-human mammals beta casein in order to obtain a food for the early infant diet for the prevention of insulin-dependent diabetes.

Further objects of the invention will be evident from the detailed  
10 description of the invention

#### Detailed description of the invention

In the attached description the amino acid sequences of importance according to the invention will be underlined. The word "substantially free" will indicate the presence of the substance (s) to which it  
15 refers in amounts ranging between 0 to 10% b.w.

The amino acid sequence of interest for the present invention is described hereafter. As mentioned above, according to the inventor there is a correlation between exposure to cow's milk and the development of insulin-dependent diabetes due to molecular mimicry  
20 between the amino acid sequences of beta casein A1 and A2 and a specific sequence of the GLUT2 protein found in the insulin-producing cells. Such a sequence has been identified as follows:

Pro-Gly-Pro-Ile-His-Asn (where the underlined sequence is SEQ ID NO:1)  
for the A1 beta casein inserted in the larger fragment: Ser-Leu-Val-  
25 Tyr-Pro-Phe-Pro-Gly-Pro-Ile-His-Asn (SEQ ID NO:3).

As already stated, such a sequence is also present in gamma casein. Other sequences corresponding to immunogenic peptides of beta casein

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which are different from those mentioned above are given as examples.  
Cow's beta casein A2 from bos taurus (63-68), Pro-Gly-Pro-Ile-Pro-Asn  
(where the underlined sequence is SEQ ID NO:2) inserted in the larger  
fragment: Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn (SEQ ID  
5 NO:4)

Beta casein from bos indicus (63-68): Pro-Gly-Pro-Ile-Pro-Asn  
(underlined sequence SEQ ID NO:2).

In comparison, human beta casein has the following sequence (48-59):  
Ser-Leu-Val-Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-Tyr (SEQ ID NO:6). The  
10 peptide fraction relevant to the present invention has been identified  
as (54-59): Val-Glu-Pro-Ile-Pro-Tyr (where the underlined sequence is  
SEQ ID NO:5). The peptide sequences of GLUT2, the glucose transporter  
inside insulin-producing beta cells in the pancreas, are the  
following:

- 15 (409-420) Ser-Phe-Phe-Glu-Ile-Gly-Pro-Gly-Pro-Ile-Pro-Trp  
(412-423) Glu-Ile-Gly-Pro-Gly-Pro-Ile-Pro-Trp-Phe-Met-Val  
(414-425) Gly-Pro-Gly-Pro-Ile-Pro-Trp-Phe-Met-Val-Ala-Glu

The inventor suggests that the sequence of A1, B and C beta casein and  
gamma casein, Pro-Gly-Pro-Ile-His (SEQ ID NO:1), and the larger  
20 fragments containing it, such as the sequences of beta casein A2, A3  
and E, Pro-Gly-Pro-Ile-Pro (SEQ ID NO:2), are responsible for the  
induction of an immune response towards beta casein which, by cross  
reactivity, would be directed towards the homologous sequence of  
GLUT2, causing damage to the cells that produce insulin.  
25 Therefore to produce a milk or in general, food product comprising  
non-immunogenic beta casein for administration in diets, particularly  
to newborns and in early infancy, would be a preventive approach

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against insulin dependent diabetes.

All caseins which do not contain the sequence Pro-Gly-Pro-Ile-His (SEQ ID NO:1) or Pro-Gly-Pro-Ile-Pro (SEQ ID NO:2) are not considered pathogenic and, therefore, can be used to produce a dietary product in

5 accordance with the present invention:

- some or all amino acids present in the above sequence are modified;

- beta casein does not contain such a sequence (e.g., it has been removed)

10 - beta casein is modified in that such a sequence is substituted with a sequence of human beta casein;

All modifications can be made by applying the well-known technique of genetic engineering and the classic biological technique of cross-selection, as described in patent WO 93/04171.

15 The milk obtained, comprising casein modified as stated above, can be administered as such or can be treated with known methods, as the casein(s) involved can be separated and used to produce food and pharmaceutical products.

In particular, the products including such casein can be used for  
20 administration in early infancy and later on as a diet for the prevention of insulin-dependent diabetes.

It is preferred that, in products according to the present invention, concentrations of A1 and/or A2 and/or other immunogenic beta caseins, in particular those with the sequence Pro-Gly-Pro-Ile-His (SEQ ID  
25 NO:1) or Pro-Gly-Ile-Pro (SEQ ID NO:2), do not represent more than 10% b.w. of the final product.

The food products of the invention can be, for instance, pasta, milk

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and milk-derived products and proteins, such as those added to food, all of which are already in the marketplace, the modification being the substitution of the immunogenic caseins with the caseins of the present invention.

- 5 Also part of the present invention are vegetable and/or synthetic proteins, such as those derived from soya. According to the teaching of the invention, it is possible to produce a pharmaceutical or food product, especially for early infancy, substantially free of beta casein, with the amino acid sequence Pro-Gly-Pro-Ile-His (SEQ ID NO:1)
- 10 or Pro-Gly-Pro-Ile-Pro (SEQ ID NO:2), or where such sequences are less than 10% of the final weight of the product. It is also possible to produce a food product or a milk according to the following alternatives:
- where the beta casein is lower than 10% b.w. or the beta casein
  - 15 comprising the amino acid sequence Gly-Pro-Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) is lower than 10% by w.
  - substantially free of beta casein comprising the amino acid sequence Gly-Pro-Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) and integrated with peptides derived from the hydrolysis of animal,
  - 20 vegetable and/or synthetic proteins, and lacking these above sequences and mixtures thereof (FR 86-00325, WO 94/06306, WO p (02539));.
  - where the beta casein comprising the amino acid sequence Gly-Pro-Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) is lower than 10% b.w. and integrated with peptides coming from hydrolysis of animal
  - 25 and/or vegetal and/or synthetic proteins lacking such sequences and mixtures thereof;
  - where the beta casein is lacking the amino acid sequence Gly-Pro-

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Ile-His (SEQ ID NO:7) or Gly-Pro-Ile-Pro (SEQ ID NO:8) in that it,has been obtained from animal species genetically not producing proteins with such sequences;

- milk naturally lacking beta casein, produced by genetically modified animals according to patent WO 93/04171;
- milk comprising human beta casein obtained from genetically manipulated microorganisms or animals, such as those described in the above mentioned patent.

The protein fractions can be derived from chemical-physical treatments of milk and from lyophilized casein, for instance by differential solubility, liquid-liquid extraction, membrane separation, chromatographic separation, as described in patents FR 86-00325 and W092/00017.

The integrations can be carried out by using recombinant beta casein produced with one of the well-known cloning methods, using yeast, bacteria, fungi or transgenic animals, such as those described in patent WO 93/04171.

A process for removing beta casein from milk is the chromatographic process, as described below.

By means of such a process the beta casein is separated, starting from acid casein, and by means of chromatography in two steps, the remaining fractions of alpha and kappa casein will be obtained.

The process can be optimized using the knowledge already available in the field. Such a process includes the use, as basic phase, of a resin of ionic exchange, for example Sepharose<sup>®</sup> from Pharmacia, with the concentration also in columns. The mobile phase is constituted by Buffer A:

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- Sodium acetate with concentration not less than 10mM;
- urea at concentration not less than 2M;
- pH between 5 and 6.

The acid casein can be dissolved in Buffer A at pH not less than 6,  
5 with the addition of a specific reducing agent, DTT, (Ditiotreeitol).  
The mixture should be left under for 24 hours, brought to pH between 5  
and 6 and placed in columns. The beta casein fraction does not  
interact with the resin and is eluted in ~~Ø~~M NaCl. It is not necessary,  
therefore, to carry out stages of increasing ionic concentration,  
10 considering that the process at hand merely involves a simple  
separation of beta casein from the other fractions, which will be  
collected in isocratic by eluting with buffer B:

- Sodium acetate at concentration no less than 10mM;
- urea at concentration no less than 2M;
- 15 - 0.8 M NaCl;
- pH between 5 and 6.

The fractions are later dialfiltrated to eliminate urea and other  
salts; after concentration, caseins are collected by acid  
precipitation and the obtained wet product is lyophilized.

20 Brief description of the drawings.

Fig. 1 is the chromatogram relating to the initial load of Example 1;

Fig. 2 illustrates a chromatographic peack relating to the beta  
casein;

Fig. 3 refers to the absence of the beta casein in the chromatogram.

25 The following examples are to be considered as illustrative of such a  
technique, therefore they should be not considered a limitation of the  
gist of the present invention.

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Example 1:

Separation of beta casein from acid casein and collection of the remaining alpha and kappa casein fractions.

Preparation

- 5 - 200 g acid casein + 3000 ml Buffer C: 20 mM sodium acetate. 4M urea, 10 mM (dithioerythritol) DTT pH 7.

Casein should be slowly dissolved in the buffer, keeping pH 7 with 2M NaOH at each addition.

Leave under stirring at 5° C for approximately 12 hours.

- 10 Filtrate the solution in pre-filter Millex AP-50 (Millipore)

Wash the prefilter with 1000 ml of Buffer C and collect.

Bring the load (4000 ml) to pH 5.5 with HCl 6M and adjust the ionic strength (2.2 mS) to 4.5 mS.

Preparational Chromatography

- 15 - FPLC Waters 600 Controller

- Revelator: Perkin Elmer UV/VIS Spectrophotometer Lambda 3B 280 nm

- Column: XK 50 Pharmacia (maximum pressure 3 bar) Ø 5 cm, height 100 cm

- Resin: S-Sepharose Pharmacia height 85 cm, volume 1670 ml

- 20 - Eluents: Buffer A Sodium acetate 20 mM

Urea 4M

pH 5.5, ionic strength 1.5 mS

Buffer B Sodium acetate 20 mM

Urea 4M

- 25 pH 5.5

Sodium chloride 1 M ionic strength 57.1 mS

All buffers are filtrated by using a Millex pre-filter AP 50 bound in

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series with filter 0.45  $\mu$ m Millipak 20 (Millipore).

- Temperature: Room temperature

- Conditioning:  $\approx$  8000 ml

Buffer A: 97% Buffer B: 3% ionic strength mix:

5 4.5 mS

Flow: 30 ml/min P=42 PSI

Time: 4 hours, 25 minutes

- Loading: 200 g acid casein dissolved in Buffer C (total  
volume 4000 ml)

10 Flow: 20 ml/min P=50 PSI

Time: 3 hours, 20 minutes

- Elution: First Stage (isocratic)  $\approx$  8000 ml

Buffer A:97% Buffer B:3%

15 Flow: 30 ml/min P=42 PSI

Time: 4 hours, 25 minutes

Second Stage (isocratic) 9000 ml

Buffer A:20% Buffer B:80%

Flow: 30 ml/min P=42 PSI

20 Time: 5 hours

#### Example 2 - Control test

An amount of the product from example 1 is tested by chromatography to evaluate the absence of beta casein in the isocratic of the second stage. Such absence is confirmed as demonstrated by the chromatogram of figure 3. By comparison in figure 1, the chromatogram relating to the initial load is illustrated, whereas in figure 2 the peak relating to the presence of beta casein only derived from the elution of the first stage is represented.

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Analytic chromatography

- 5 - HPLC Perkin Elmer Biocompatible Binary Pump 250
- Revelator: Perkin Elmer LC95 280 nm
  - Column: Mono S HR 5/5 Pharmacia
  - Loop: 100 µl
  - Eluents:     Buffer A\*     Sodium acetate 20 mM  
                              Urea 6 M  
                              pH 5
  - Buffer B\*     Sodium acetate 20 mM  
                              Urea 6 M  
                              Sodium chloride 1 M  
                              pH 5
- 10 - Temperature: Room temperature
- Flow: 1 ml/min
  - Conditioning: 8'00"     Buffer A\* 100%   Buffer B\* 0%
  - Elution: gradient 5'00"     Buffer B\* 50%   Buffer B\* 50%  
  (increase of B\* 1% min)
  - isocratic 2'00"     Buffer A\* 50%   Buffer B\* 50%
- 15   isocratic 5'00"     Buffer A\* 0%     Buffer \* 100%

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### Example 3

The product of Example 1 has been purified from urea by the following method of diafiltration.

- Ultrafiltration S.G.I.

5 - Cellulose membrane S-10 10.000 Da Amicon

- Buffer of dialysis: demineralized water  
sodium acetate 10 mM pH 7  
ionic strength 0.8 mS  
total volume 250 l (5 washes)

- Permeate flow: 32+37 l/h

- Temperature: 10°C

- Product concentration 50 l up to 20 l

10 The product has been tested to verify the absence of urea as follows:

#### Urea Test

- UV method (Boehringer Mannheim)

- Spectrophotometer: Lambda 3B 340 nm (Perkin Elmer)

Reagents	Blank	Sample
Solution 1	1.00 ml	1.00 ml
Sample solution	---	0.10 ml
Solution 2	0.02 ml	0.02 ml
Bidistilled water	2.00 ml	1.90 ml

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Incubate 5' at 20-25°C; read the absorbance (A1)

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Solution 3	0.02 ml	0.02 ml
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Incubate 20' at 20-25°C; read the absorbance (A2)

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Solution 1 = Triethanolamin buffer, pH 8.2 oxoglutarate, NADH  
Solution 2 = Urase  
Solution 3 = Dehydrogenase glutamate

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The lyophilization is carried out on the product free of urea, by using a Christ model Beta 1-16 equipment.

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